Young Investigator Award Lecture
Will be published in J. Angiology

GENERATION OF FREE RADICALS DURING COLD INJURY AND REWARMING

Jaisimha Iyengar, M.D., Saswati Samanta, Anna George, M.D., John C. Russell, M.D.,

and Dipak K. Das, Ph.D.

Surgical Research Center, Department of Surgery

University of Connecticut School of Medicine

Farmington, Connecticut 06032

Contract N00014-88-K-0546

Support Sources: ~~Grants from~~ the United States Department of the Navy

(1172-000-22-20111) and USPHS HL22559.

Running Head: Free Radicals in Cold Injury

ABSTRACT

Cold injury is often associated with irreversible cell damage. However, to date the pathophysiology of cold injury is not known. The present study examines the mechanism of such injury. New Zealand white rabbits were anesthetized with ketamine and xylazine, and then ventilated using a Harvard ventilator. The femoral artery and vein were exposed. A flow probe was placed around the femoral artery, which in turn connected to a six-channel recorder. A continuous display of electrocardiogram was obtained on Lead II by connecting the limb leads to the same recorder. A thermocouple probe was inserted inside the leg to be cooled. After baseline measurements, one leg was cooled with a freezing mixture up to 0°C, which was followed by rewarming; the other leg served as control. During the experiment, blood samples were withdrawn from the femoral artery for the subsequent analysis of creatine kinase (CK), lactate dehydrogenase (LDH), and malonaldehyde (MDA). At the end, salicylate was injected through the femoral vein to trap any hydroxyl radical (OH·) formed. Rabbits were immediately sacrificed, and biopsies were withdrawn and frozen at liquid N_2 temperature to analyze OH·. Our results indicated that local blood flow in the cold-exposed leg was reduced significantly, suggesting that cold injury was associated with ischemic insult. CK and LDH were increased after cold exposure, then increased further during rewarming. MDA formation followed a similar pattern. OH· generated after cooling increased significantly upon rewarming. These results indicate that rewarming is associated with an episode of ischemia/reperfusion, with simultaneous generation of free radicals which, at least in part, may be responsible for cellular injury associated with rewarming.

INTRODUCTION

Cold injury is often associated with irreversible cell damage[1,2]. However, to date the pathophysiology of cold injury is not known. During prolonged exposure to cold, intracellular water moves to the interstitial space, and the cells become hyperosmolar and then freeze[3]. In addition, the hemoglobin dissociation curve is shifted to the left, thereby preventing release of O_2 to the tissue[4]. The poor perfusion of capillaries in conjunction with decreased ability to release O_2 may induce severe ischemic insult to the affected tissues despite the fact that hypothermia reduces the rate of cellular metabolism significantly.

During rewarming, with the shifting of the hemoglobin dissociation curve to the original position in concert with reinstitution of perfusion, the tissue is relieved from the ischemic insult. Although the phenomenon has never been explored in conjunction with cold injury, it is quite possible that cold-induced ischemic tissue is subjected to the so-called "reperfusion injury" during the rewarming phase. Current research has pointed to the "reperfusion injury", which presumably amplifies ischemic injury and is clearly distinct from the injury associated with ischemia[5]. A role of free radicals has been indicated in the pathogenesis of reperfusion injury in many tissues[6-9].

This study was designed to explore the possibility of the occurrence of reperfusion injury after cold injury. Our results indicated that cold injury was associated with an ischemic episode. Oxygen-derived free radicals were formed during rewarming, resulting in further tissue damage as frequently observed in "reperfusion injury".

MATERIALS AND METHODS

Animal Preparation

New Zealand white rabbits of about 2.5 kg body weight were anesthetized with xylazine (5 mg/kg) and ketamine (30 mg/kg) and were maintained under anesthesia during the entire experiment. An electric clipper was used to remove hair from the hind limb as well as from the front of the neck area. A tracheostomy was performed, and the rabbits were ventilated by a Harvard ventilator (15 ml volume and 50 strokes/min). Femoral arteries and veins of both sides were exposed and dissected free

of tissue. A flow probe (Transonic, Ithaca, NY) was placed around the femoral artery, which in turn was connected to a six-channel simultrace chart recorder (Honeywell Inc., Pleasantville, NY). The femoral vein of the same side was cannulated with an I.V. placement catheter for withdrawal of blood samples. Another such catheter was placed in the femoral artery of the other limb. A continuous display of electrocardiogram (EKG) was obtained on Lead II by connecting the limb leads to the same recorder. The baseline values were established by measuring EKG and blood flow, as well as by estimating the arterio-venous difference of creatine kinase (CK), lactate dehydrogenase (LDH), and malonaldehyde (MDA) from the blood samples. One of the legs was then cooled down to 0°C with a freezing mixture containing ice and salt. Continuous monitoring of the interstitial temperature was achieved by inserting a thermocouple probe (Omega Engineering, Inc., Stamford, CT) into the limb. Tissue temperature was maintained for 20 min at 0°C. The ice was then removed, and the limb was allowed to rewarm to room temperature. During the experiment, blood samples were withdrawn at regular intervals of time from both the vein and artery for the subsequent assay of CK, LDH, and MDA formation. Blood flow was also continuously monitored. At the end of the experiment, salicylate (2 mM) was injected through the femoral artery to trap free radical. The rabbits were immediately sacrificed by an overdose of sodium pentobarbital. Tissue biopsies were withdrawn for assay of $OH^{\cdot}$.

Assay for OH

The method used to trap and quantitate OH was similar to that described by Grootveld and Halliwell[10]. The tissue was homogenized under liquid N_2. The ground tissue was suspended in a buffer containing a mixture of sodium citrate (0.05 M) and sodium acetate (0.03 M) (pH 4.5); 50 μl of 70% perchloric acid was then added to the mixture. The resultant mixture was degassed and filtered through a Rainin Nylon-66 membrane filter (0.45 μM). The sample (20 μl) was injected onto an Altex Ultrasphere 3 μODs (75 x 4.6 mm) equipped with a Water Associates HPLC unit consisting of a Model 510 pump and a Model 460 electrochemical detector. The hydroxylated products of salicylic acid were eluted with buffer (degassed and filtered) containing 0.03 M sodium acetate and 0.05 M sodium citrate

(pH 4.5) at a flow rate of 0.8 ml/min. The detector potential was maintained at 0.6 V, employing Ag/AgCl reference electrode.

Measurement of Lipid Peroxidation

Malonaldehyde was measured as an index for lipid peroxidation. Plasma (0.5 ml) was added to 0.5 ml ice-cold perchloric acid (15%) and then treated with 0.75% thiobarbituric acid (TBA) as described previously[11]. Samples were boiled for 20 min and centrifuged to remove the pellet. The color of the supernatant was read at 535 nm. The concentration of MDA (nmol/ml) was calculated by using a molar extinction coefficient of 156 mM^{-1} cm^{-1}.

Assay for CK and LDH

CK and LDH were assayed in plasma samples obtained from the femoral artery, using an assay kit obtained from Sigma Chemical Company (St. Louis, MO) as described elsewhere[12].

Statistical Analysis

All measurements are expressed as mean values ± SEM. Student's t-test or two-tailed t-test was used for comparison of the data between two groups or within each group for each variable. For multigroup comparisons, analysis of variance was used. For those variable and time points at which the groups were not equal, multiple comparison methods were used to establish all possible pair-wise comparisons to better identify which groups differed from the others. Differences were considered significant when the *p* value was less than 0.05. Each point shown in Figures 1-7 is the mean value of at least six different experiments in each group.

RESULTS

Monitoring of Heart by EKG

The electrocardiographic pattern showed regularity in rate and rhythm of heart during the entire period of cooling the leg from 28°C to 0°C (Fig. 1). Heart rate was maintained at a steady condition even during rewarming after keeping the limb at 0°C for 20 min.

Blood Flow During Cooling and Rewarming

Hemodynamic changes in the femoral artery were continuously monitored with the flow probe. The flow pattern was also recorded during the experiment (Fig. 2). Under normal conditions, the flow rate was about 3.5 ml/min. During cooling of the limb, the flow rate continuously dropped. By the time the interstitial temperature became 0°C, it decreased to about 28% of the normal value to 1 ml/min (Fig. 3). A steady low flow rate was maintained for the rest of the cooling period. When rewarming was initiated by removing the freezing mixture from the leg, the flow rate gradually increased even when the interstitial temperature remained at 0°C. The flow rate continued to rise, and at the end of the rewarming th : flow rate was completely restored.

Release of LDH and CK

The release of arterial plasma LDH is plotted against temperature during cooling and rewarming of the rabbit leg (Fig. 4). There was no change in plasma LDH levels during cooling from 30°C to 15°C, but below that temperature there was a slight increase in LDH release. A remarkable increase in plasma LDH (about 2-fold) was noted at the end of rewarming, suggesting that tissue damage occurred mostly during the rewarming period. CK, another marker for tissue necrosis, followed a similar pattern (Fig. 5). After cooling, a slight increase in CK activity was noticed, but the differences were not statistically significant. During rewarming, however, CK increased dramatically. At the end of reperfusion, these values were 2.5-fold higher compared to the baseline levels.

Estimation of Lipid Peroxidation

Malonaldehyde formation, a presumptive marker for lipid peroxidation, remained unchanged during cooling (Fig. 6). During rewarming, MDA formation increased significantly and reached a 1.4-fold higher value compared to control at the end of the rewarming phase. This suggests that lipid peroxidation occurred only during rewarming of the cooled tissue.

Estimation of Free Radical by HPLC

In some experiments, salicylate was injected through the femoral vein to trap any $OH^{\cdot}$ radical which might be generated in the tissue. We assayed the $OH^{\cdot}$ prior to cooling and at the onset of

rewarming, when ice was removed from the leg and blood flow began to rise. We choose this point because oxygen-derived free radicals are known to be produced at the onset of reperfusion of an ischemic organ. The results are shown in Figure 7. The OH· signal increased about 3-fold in the cooled tissue (after ice was removed, when blood flow began to rise, as shown in Fig. 3) compared to baseline control values (Fig. 6B compared to 6A).

DISCUSSION

The results of our study demonstrate for the first time that free radicals are formed during rewarming of cooled tissue. The results also suggest that the cooling/rewarming episode may undergo similar pathophysiologic changes as those usually associated with ischemia/reperfusion of tissues. This gives rise to a novel and interesting hypothesis concerning the pathophysiology of cold injury. O· results clearly indicate that cellular injury mediated by cold exposure was amplified during rewarming, suggesting occurrence of a phenomenon similar to that of so-called "reperfusion injury" which is associated with reperfusion of ischemic tissues[13,14].

In the present study, we have clearly demonstrated that hypothermic insult resulted in reduction of blood flow, which dropped to 28% of the original value after cooling to 0°C. This value was almost restored after rewarming. We chose to hold the leg at 0°C up to a period of 20 min, because in this model, holding the temperature beyond 20 min resulted in only partial recovery of blood flow during rewarming. This suggests some irreversible injury during prolonged hypothermic insult. Indeed, it is known that poor perfusion in conjunction with anoxia and flow through cold-injured vessels can cause aggregation of red cells, platelets, and polymorphonuclear leukocytes (PMN) leading to thrombosis and tissue infarction, which ultimately may lead to gangrene formation[15].

Cold injury resulted in significant damage to the cells as evidenced by the enhanced release of CK and LDH. The levels of CK and LDH were further enhanced during rewarming, suggesting further damage to the cell during this period.

Development of oxidative stress was indicated by the increased amount of MDA formation (presumptive marker for lipid peroxidation) during cooling. There is evidence in the literature regarding

the development of oxidative stress in cold-induced ischemic damage. For example, it is general practice to cool the heart to 4°C and induce cardiac arrest by hypothermia during open-heart surgery. Increased lipid peroxidation and glutathione release from heart has been reported during hypothermic arrest[6,16]. Oxidative stress has also been cited as a probable cause for the deteriorating function in long-term perfused kidneys[17]. In this study, MDA formation was continuously increased in cortex samples as hypothermic storage time lengthened. Studies with liver cells demonstrated that isolated hepatocytes stored as a suspension in ice for 24 hours form more MDA compared to those from freshly isolated cells[18]. Our study supports these previous observations and further demonstrates the presence of $OH^{\cdot}$ in cold-injured tissue.

The amount of $OH^{\cdot}$ was further elevated during rewarming, suggesting development of increased oxidative stress. This result correlated with the formation of enhanced MDA during rewarming. Further cellular injury was also observed, as evidenced by increased LDH and CK release from tissues after rewarming. Taken together, these results suggest the development of "rewarming injury" similar to that observed during reperfusion of ischemic tissues. The result of our study further suggests that oxygen-derived free radicals may be responsible for such injury.

However, our results did not demonstrate the mechanism of free radical generation, nor did they indicate any potential source of free radicals in the cold-rewarmed tissue. However, several possible sources may be considered. For example, the depletion of ATP during ischemia would cause generation of hypoxanthine and xanthine, which might react with xanthine oxidase present in tissue to generate oxygen-derived free radicals[19]. In addition, PMN may be activated during cold injury and may cause generation of oxy radicals by well-known mechanisms[20]. Whatever the source of injury may be, our results clearly demonstrate increased formation of $OH^{\cdot}$ in the rewarmed leg. $OH^{\cdot}$ may be formed during the incomplete reduction of molecular oxygen according to the iron-catalyzed Fenton reaction[21]:

$$O_2 + e^- \longrightarrow O_2^- \quad \text{----- (i)}$$

$$2O_2^- + 2H^+ \longrightarrow O_2 + H_2O_2 \quad \text{----- (ii)}$$

$$O_2^- + H_2O_2 \longrightarrow O_2 + OH^- + OH\cdot \quad \text{----- (iii)}$$

The HPLC technique to trap OH· (as described in this study) is a unique way to establish the presence of OH·. When salicylic acid is allowed to react with OH·, the hydroxylated products formed are extremely stable and can be detected by HPLC[10]. In order to demonstrate that the signal was indeed due to the formation of OH·, we used a specific OH· scavenger such as dimethylsulfoxide (DMSO) to scavenge the OH· signal. The presence of any OH· signal described in this study was always confirmed by its scavenging with DMSO.

In summary, our study demonstrated for the first time the occurrence of "rewarming injury" associated with rewarming of cold tissue. This rewarming injury is similar to that observed in case of reperfusion injury. The increased presence of OH· as observed in the cold/rewarmed tissue suggests a role of oxygen-derived free radicals in the "rewarming injury."

Address correspondence to:

Dipak K. Das, Ph.D.

Cardiovascular Division, Department of Surgery

University of Connecticut School of Medicine

Farmington, Connecticut 06032

ACKNOWLEDGEMENTS:

This study was supported by grants from the Office of United States Naval Research (USA RR04108) and NIH (HL33889). The technical assistance of Ms. Saswati Samanta is gratefully acknowledged, as is the skillful secretarial assistance of Mrs. Joanne D'Aprile.

REFERENCES

1. Bourne GH, Piepkorn MW, Clayton F, *et al.* Analysis of microvascular changes in frostbite injury. J Surg Res **40**:26-35, 1986.

2. Bowers WD, Habbard RW, Daum RC, *et al.* Ultrastructural studies of muscle cells and vascular endothelium immediately after freeze-thaw injury. Cryobiology **10**:9-21, 1973.

3. Gadarowski JJ Jr, Esce JD. Acute systemic change in blood cells, protein coagulation, fibrinolysis, and platelet aggregation after frostbite injury in the rabbit. Cryobiology **21**:359-370, 1984.

4. Mundth E. Studies on the pathogenesis of cold injury. **In**: Viereck E, ed. Proceedings of the Symposium on Arctic Medicine and Biology: IV Frostbite. Fort Wainwright, AL: Arctic Aero-medical Laboratory, 1964.

5. Smith HJ, Kent KM, Epstein SE. Contractile damage from reperfusion after transient ischemia in the dog. J Thorac Cardiovasc Surg **75**:452-457, 1978.

6. Das DK, Engelman RM, Rousou JA, *et al.* Pathophysiology of superoxide radical as potential mediator of reperfusion injury in pig heart. Basic Res Cardiol **81**:155-166, 1986.

7. Parks DA, Berkley GB, Granger DN, *et al.* Ischemic injury in the cat small intestine. Role of superoxide radicals. Gastroenterology **82**:9-15, 1982.

8. Manson PN, Anthelli RM, Im NJ, *et al.* The role of oxygen-free radicals in ischemic tissue injury in island skin flaps. Ann Surg **198**:87-90, 1983.

9. Jolly SR, Kane WJ, Bailie MB, *et al.* Canine myocardial reperfusion injury. Its reduction by combined administration of superoxide dismutase and catalase. Circ Res **54**:277-285, 1984.

10. Grootveld M, Halliwell B. Aromatic hydroxylation as a potential measure of hydroxyl radical formation *in vivo*. Biochem J **237**:499-504, 1986.

11. Prasad MR, Clement R, Otani H, *et al.* Improved myocardial performance induced by clofibrate during reperfusion after acute myocardial infarction. Can J Physiol Pharmacol **66**:1518-1523, 1988.

12. Das DK, Flansaas D, Engelman RM, *et al.* Age related development profiles of the antioxidative system and the peroxidative status of the pig heart. Biol Neonate **51**:156-169, 1987.

13. Otani H, Engelman RM, Rousou JA, *et al.* The mechanism of myocardial reperfusion injury in neonates. Circulation **76**:V161-V167, 1987.

14. Gauduel Y, Duvelleroy MA. Role of oxygen radicals in cardiac injury due to reoxygenation. J Mol Cell Cardiol **16**:459-470, 1984.

15. Bangs CC. Hypothermia and frostbite. Emerg Med Clin North Am **2**:475-487, 1984.

16. Otani H. Cardiac performance during reperfusion improved by pretreatment with oxygen free radical scavengers. J Thorac Cardiovasc Surg **91**:290-295, 1986.

17. Belzer FO, Hoffman R, Southard J. Aerobic and anaerobic perfusion of canine kidney with a new perfusate. In: Pegg DE, Jacobson JA, Halasz N, eds. Organ Preservation: Basic and Applied Aspects. Lancaster:MTP Press, 1982:253-259.

18. Lund P, Farrell R. Free radicals appear to affect survival of hepatocytes at 4°C. Free Rad Res Commun **1**:69-75, 1985.

19. McCord J. Oxygen derived free radicals in postischemic tissue injury. N Engl J Med **312**:159-163, 1985.

20. Lucchesi BR, Mullane KM. Leukocytes and ischemia induced myocardial injury. Ann Rev Pharmacol Toxicol **26**:201-224, 1986.

21. Kellogg EW, Fridovich I. Superoxide, hydrogen peroxide, and singlet oxygen in lipid peroxidation by a xanthine oxidase system. J Biol Chem **250**:8812-8817, 1975.

FIGURE LEGENDS

Figure 1. Electrocardiogram of rabbit heart on lead II by connecting the limb leads to simultaneous chart recorder using a chart speed of 25 mm/sec.

(A) Under normal conditions at room temperature.

(B) During cooling of the leg at O°C.

(C) During rewarming period at 15°C.

Figure 2. Changes in the pattern of blood flow waves in the femoral artery of rabbit leg using an ultrasonic flow probe.

(A) Under normal conditions at room temperature.

(B) During cooling of the leg at 0°C.

(C) During rewarming period at room temperature.

Figure 3. Changes in the blood flow rate (ml/min) in the femoral artery of rabbit leg during cooling and rewarming. Blood flow was recorded using an ultrasonic flow probe.

Figure 4. Changes in the level of lactate dehydrogenase in plasma obtained from the femora artery of rabbit leg during cooling and rewarming.

Figure 5. Changes in the level of creatine kinase in plasma obtained from the femoral artery of rabbit leg during cooling and rewarming.

Figure 6. Changes in the level of malonaldehyde in plasma obtained from the femoral artery of rabbit leg during cooling and rewarming.

Figure 7. Chromatograms showing generation of $OH^{\cdot}$ in tissue biopsies obtained from leg. Salicylic acid was injected through the femoral vein, and then biopsies were withdrawn and immediately frozen under liquid nitrogen as described in Methods. Chromatograms were obtained with an HPLC using an electrochemical chemical detector.

A. Control tissue (pre-cooling).

B. Cooled tissue at the onset of rewarming.

FIGURE 1

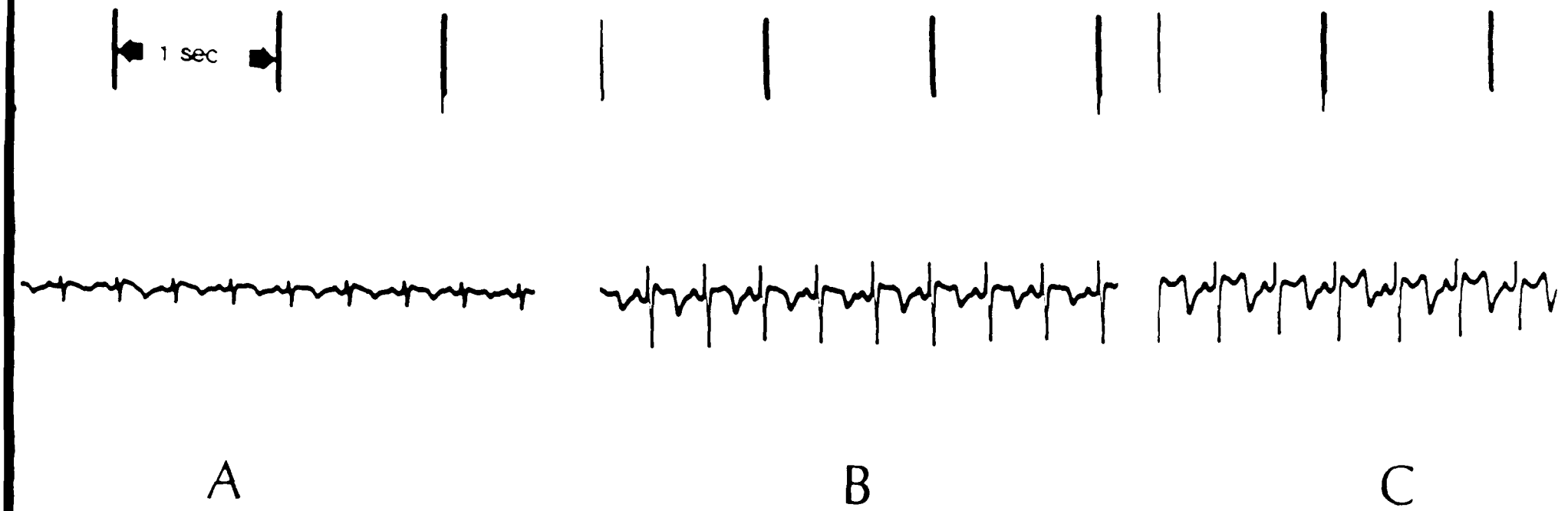


FIGURE 2

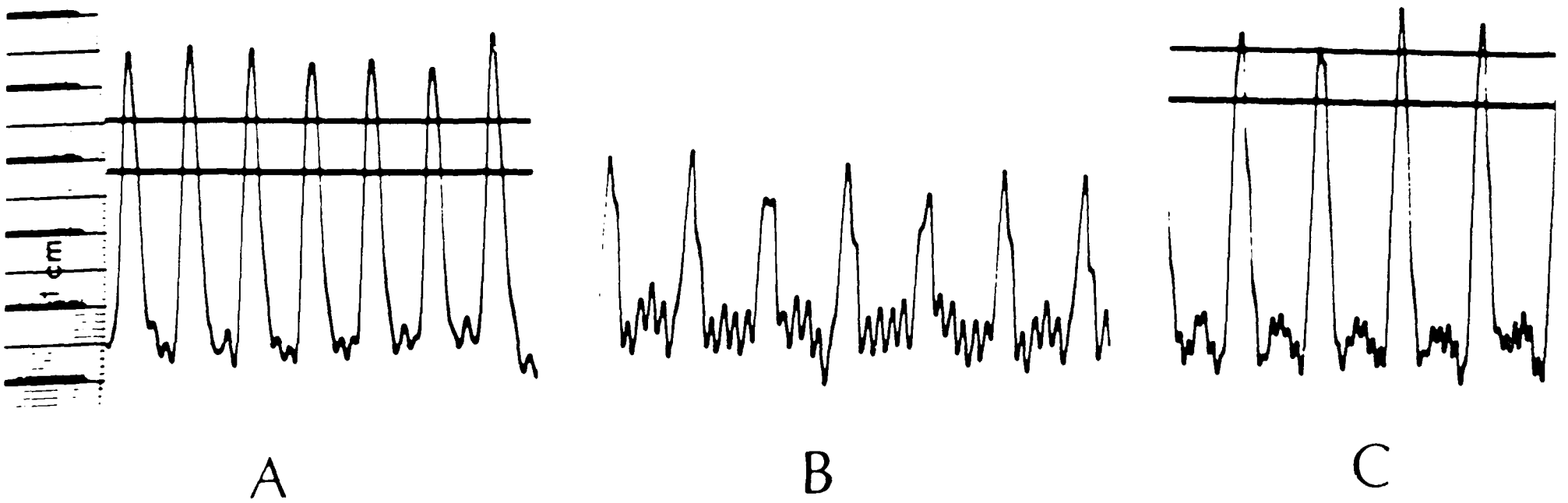


FIGURE 3

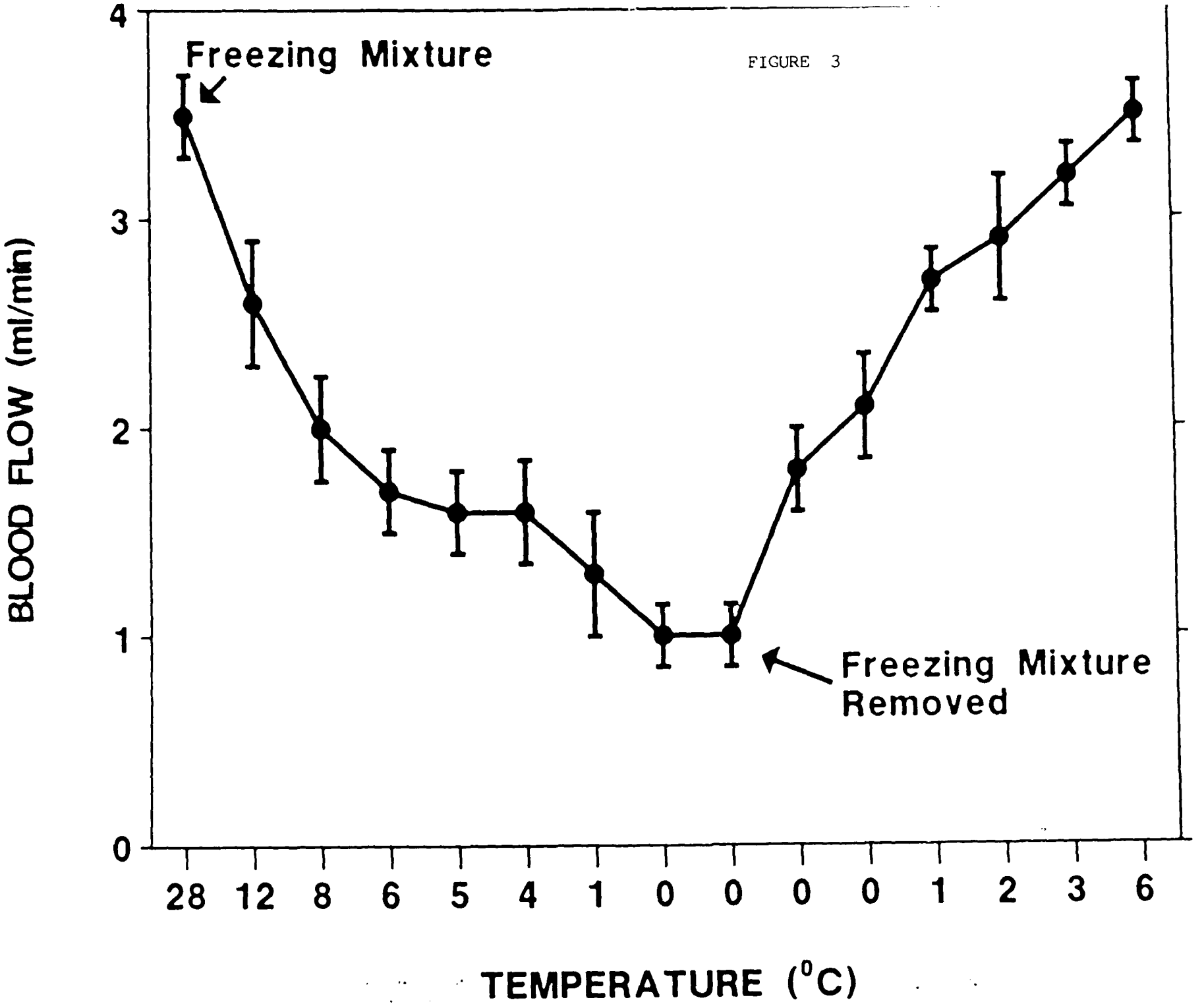

Freezing Mixture
Freezing Mixture Removed
BLOOD FLOW (ml/min)
0
1
2
3
4
28 12 8 6 5 4 1 0 0 0 0 1 2 3 6
TEMPERATURE (°C)

FIGURE 4

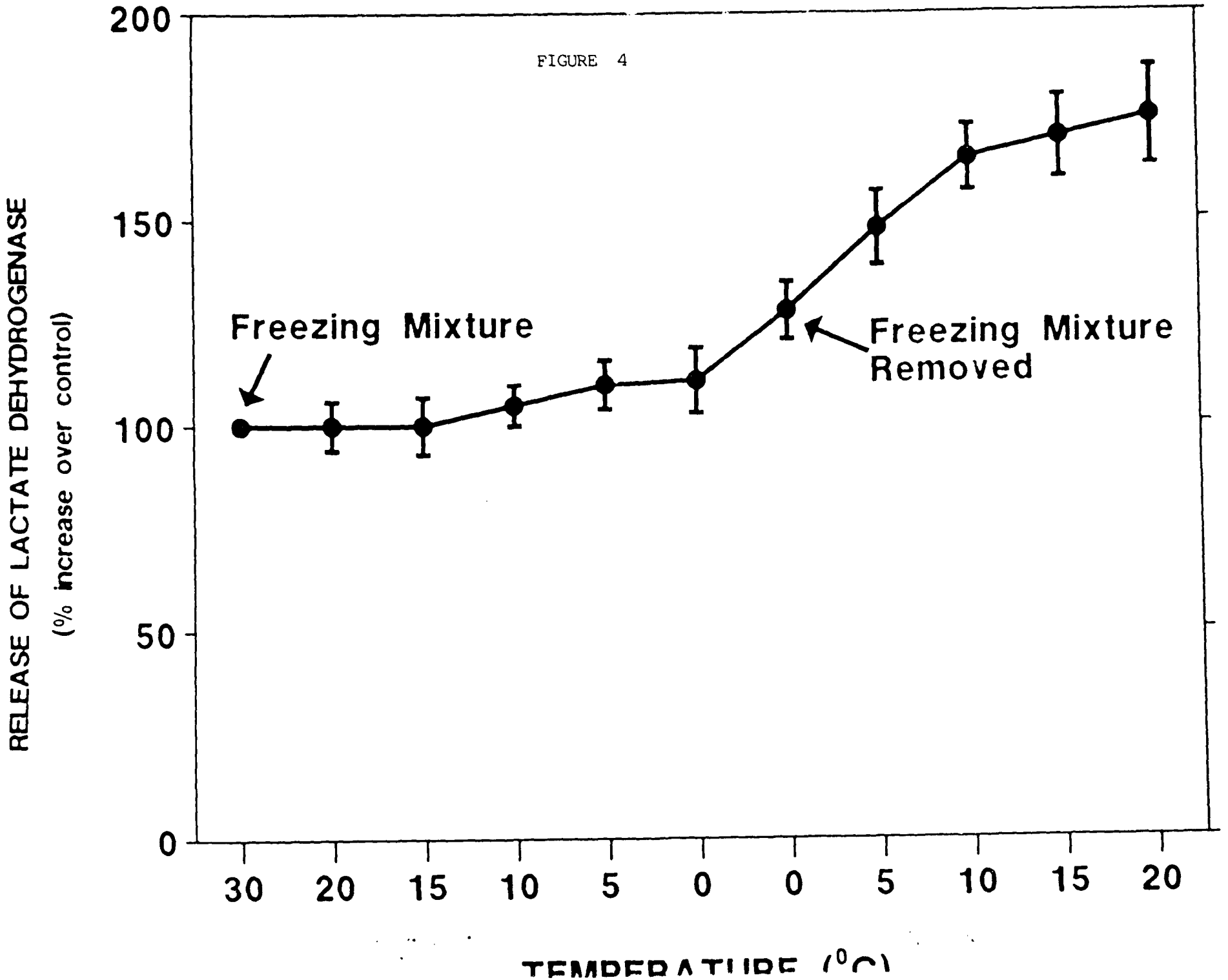

200
150
100
50
0
RELEASE OF LACTATE DEHYDROGENASE
(% increase over control)
Freezing Mixture
Freezing Mixture
Removed
30
20
15
10
5
0
0
5
10
15
20
TEMPERATURE (°C)

FIGURE 5

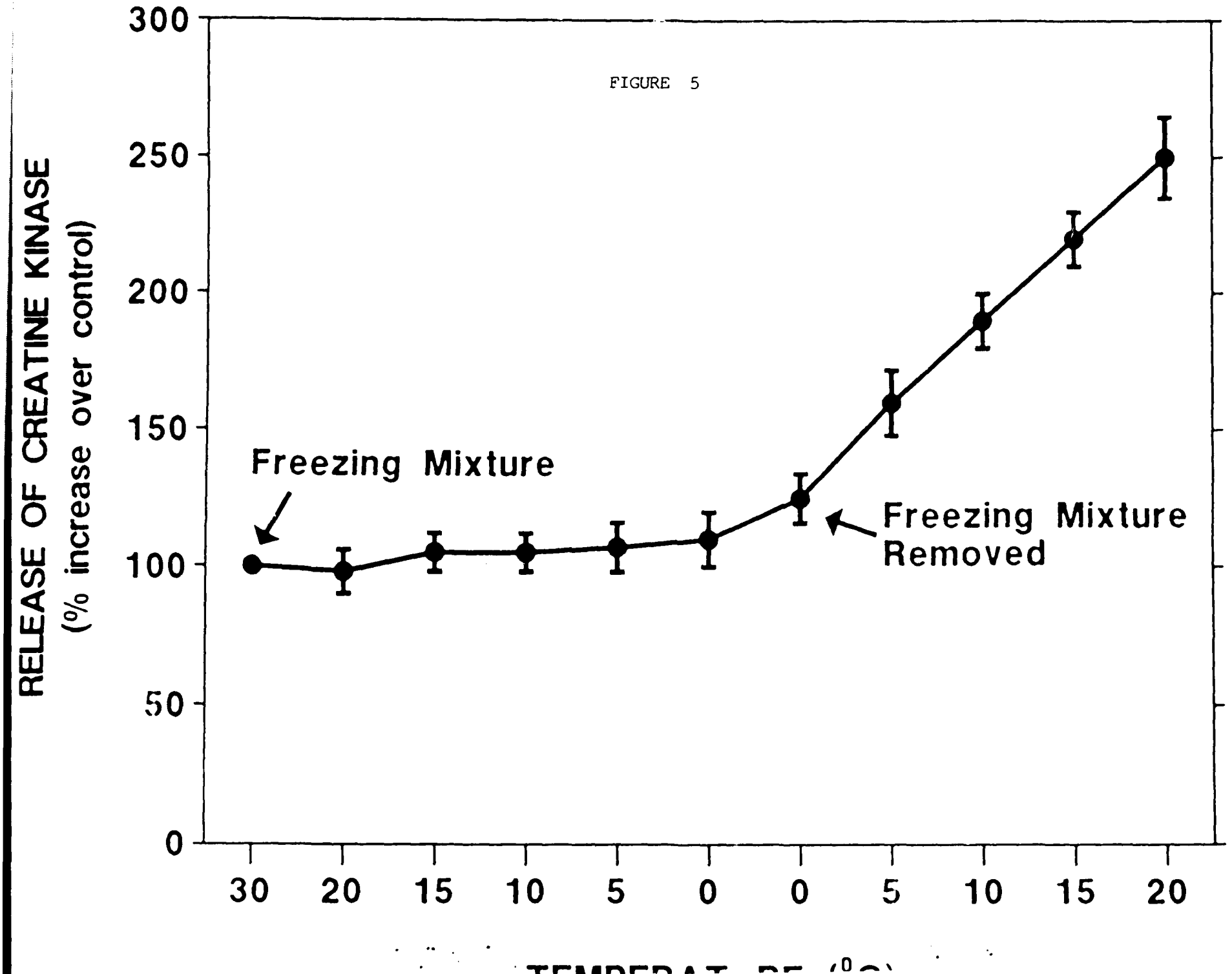

RELEASE OF CREATINE KINASE
(% increase over control)
300
250
200
150
100
50
0
Freezing Mixture
Freezing Mixture
Removed
30
20
15
10
5
0
0
5
10
15
20

FIGURE 6

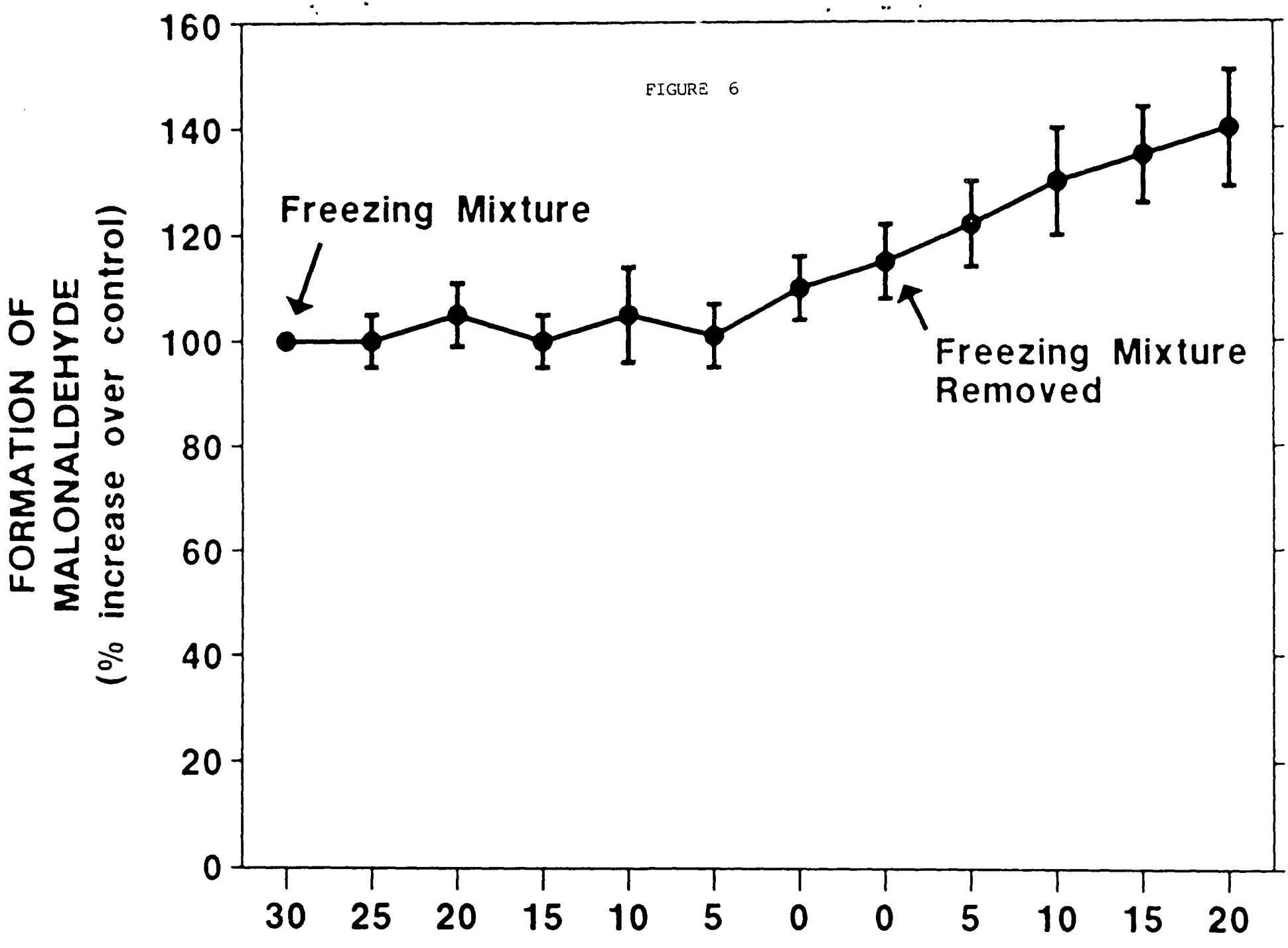
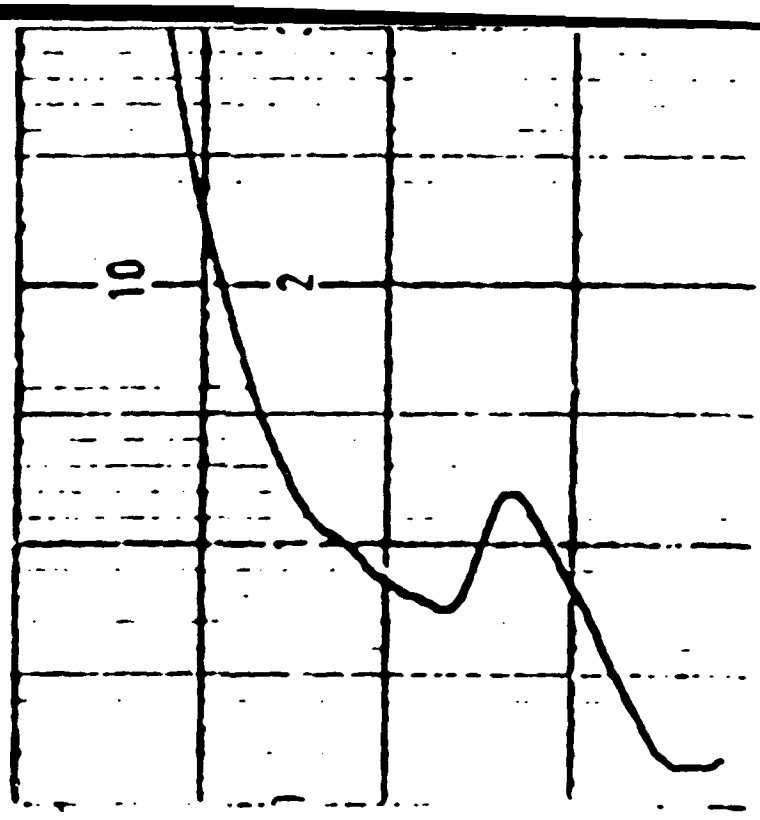
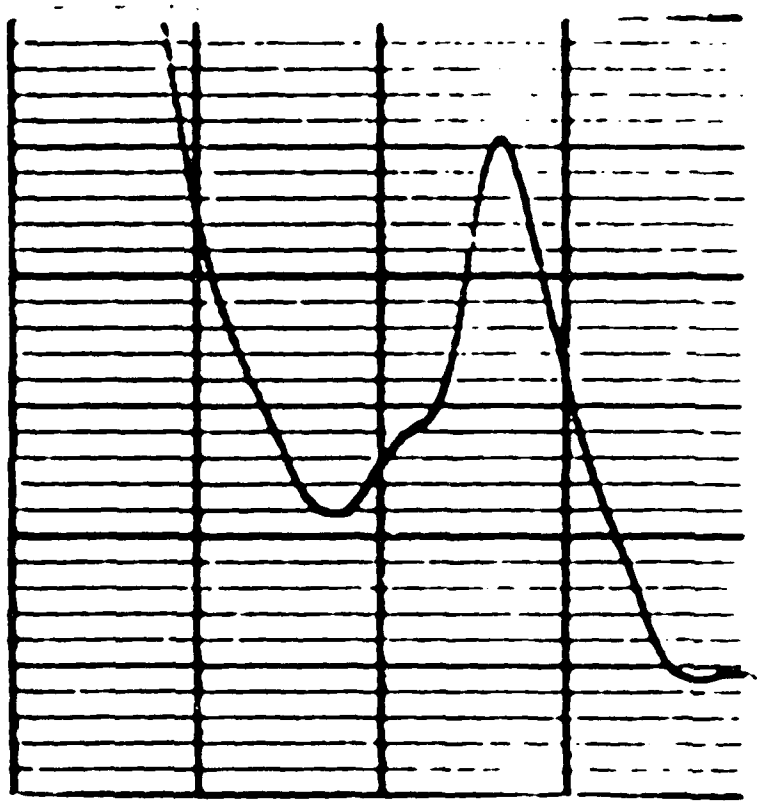

FORMATION OF MALONALDEHYDE
(% increase over control)
160
140
120
100
80
60
40
20
0
Freezing Mixture
Freezing Mixture Removed
30
25
20
15
10
5
0
0
5
10
15
20

FIGURE 7



A



B